DNA/polyethylenimine transfection particles: Influence of ligands, polymer size, and PEGylation on internalization and gene expression

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ABSTRACT Receptor-binding ligands have been incorporated into DNA/polyethylenimine (PEI) complexes to enhance cell binding and cellular internalization. This study characterizes receptor-mediated uptake of DNA/PEI complexes on a cellular basis. A novel assay based on flow cytometry was applied, discriminating between total cell-associated and extracellularly bound DNA complexes. Receptor-mediated uptake of ligand-containing DNA/PEI (molecular weight, 800 kd) complexes was found to occur quickly (within 1 hour), whereas unspecific uptake through adsorptive endocytosis is less efficient or requires extended periods to reach the same degree of internalization. Rapid, receptor-mediated internalization requires a small complex size; however, large, aggregated complexes show higher gene expression. Using PEI 25 kd conjugated to large proteins such as transferrin or antibodies, improper condensation with DNA leads to suboptimal uptake and gene expression, whereas partial replacement of ligand-PEI with unconjugated PEI increases both uptake and transfection. In contrast, the 8 kd protein epidermal growth factor conjugated to PEI 25 kd properly condenses DNA and mediates specific uptake into human adenocarcinoma (KB) cells. Modification of the complex surface with appropriate amounts of poly(ethylene glycol) (PEG) does not block ligand-mediated internalization. A higher degree of PEGylation reduces the internalization of transferrin or antibody-containing complexes to a level similar to that of ligand-free complexes. In contrast, epidermal growth factor-mediated uptake is less effected by excessive PEGylation.

KEYWORDS: gene transfer, receptor mediated endocytosis, polyethylenimine, flow cytometry, poly(ethylene glycol)

INTRODUCTION

Gene transfer via receptor-mediated endocytosis can provide several advantages for selective targeting of gene transfer particles to certain cell types. Several ligands have been incorporated into cationic DNA carriers to enhance transfection efficiency in vitro or in vivo, such as asialoorosomucoid^{1,2} or sugar residues³⁻⁵ for hepatocytes, transferrin for a wide subset of cells^{6,7}, antibodies⁷⁻⁹, or growth factors^{10,11}. In these cases, the level of reporter gene expression was used to measure the efficiency of receptor-mediated gene transfer, whereas the uptake was measured by qualitative microscopical methods. Recently we described a rapid method of measuring simultaneous binding to the cell surface and internalization of DNA complexed with cationic carrier molecules¹². In the current study we applied this assay for the study of receptor-mediated uptake of ligand-containing DNA/polyethylenimine (PEI) complexes, which were previously identified as potent transfection reagents^{7,13,14}. Uptake was evaluated for 3 different ligands: transferrin (Tf) as ligand for the Tf receptor of different tumor cell lines, antibody OKT3 for targeting the CD3 receptor of a T-cell lymphoma cell line (Jurkat), and epidermal growth factor (EGF) for targeting a human adenocarcinoma cell line (KB). We also studied the influence of DNA complex size, molecular weight of the PEI used, and modification of particle surface with poly(ethylene glycol) (PEG) on cell binding, internalization, and reporter gene expression.

MATERIALS AND METHODS

Chemicals and conjugates

The nucleic acid stains YOYO-1 and TOTO-3 (1 mM stock solution in dimethylsulfoxide [DMSO]) were obtained from Molecular Probes (Leiden, the Netherlands). PEI 25 kd (PEI₂₅) was obtained

from Aldrich (Vienna, Austria). PEI 800 kd (PEI₈₀₀) was obtained from Fluka (Buchs, Switzerland). The solutions were titrated with HCl solution to a pH of 7.4 and used as a 9 mg/mL stock and 1 mg/mL working solution. Human transferrin (iron free) was obtained from Biotest Pharma (Dreieich, Germany). A monoclonal antibody directed against human CD3 was purified from supernatants of the hybridoma cell line OKT3 (obtained from ATCC, Rockville, MD; catalog number CRL 8001). Mouse EGF was obtained from Serotec (Oxford, UK). Synthesis of Tf-PEI and antiCD3-PEI conjugates was described in Kircheis et al.^{7,15}. Synthesis of EGF-PEI₂₅ has been performed as described¹⁶. The following conjugates have been used: Tf-PEI₈₀₀ (Tf/PEI 2/1 or 3/1 mol/mol), Tf-PEI₂₅ (Tf/PEI 1/1 mol/mol), antiCD3-PEI₂₅ (1/1.9 mol/mol), and EGF-PEI₂₅ (1.25/1 mol/mol). M-PEG-SPA 5000 with an average molecular weight of 5000 d was obtained from Shearwater Polymers (Huntsville, AL) and used as a 20 mg/mL stock solution in water-free DMSO. Collagenase 1A (crude collagenase from *Clostridium histolyticum*, 347 U/mg) was obtained from Sigma (Vienna, Austria).

Cell culture

K562 cells (human erythromyeloid leukemia, ATCC CCL-243) were cultured in Roswell Park Memorial Institute (RPMI) medium 1640, 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics. Jurkat cells (human acute T-cell leukemia; Jurkat clone E6-1; ATCC TIB 152) were cultured in RPMI as K562 cells plus 20 mM HEPES pH 7.4. KB cells (human epidermoid adenocarcinoma; ATCC CCL-17) were cultured in Dulbeccos Modified Eagle medium (DMEM) with 10% FCS, 2 mM glutamine, and antibiotics. For transfection and internalization experiments, 2×10^5 KB cells per well were seeded in 6 well plates (35-mm diameter, Costar, Acton, MA) in 2.5 mL DMEM 1 day before incubation with complexes. K562 and Jurkat cells were seeded in 48 well plates 1 to 3 hours before transfection (2×10^5 cells in 0.2 mL RPMI containing 20 mM HEPES pH 7.4). Internalization and transfection studies were carried out in the same media used for cultivation (including 10% FCS).

Complex formation and PEGylation

Plasmid DNA was fluorescently labeled with the intercalating nucleic acid stain YOYO-1 using a molar ratio of 1 dye molecule per 300 base pairs for 30 minutes at room temperature (RT) in the dark. The labeled plasmid DNA (pCMV-Luc) was complexed with ligand-PEI or PEI in either HEPES buffered saline (HBS) or HEPES buffered glucose (HBG) with a final DNA concentration of 20 µg/mL as described¹⁷. Thirty minutes after complex formation, complexes were PEGylated with the appropriate amount of PEG for 1 hour at RT as described¹⁸.

Flow Cytometry Analysis

Suspension cell lines (K562, Jurkat) were washed once with Phosphate Buffered Saline (PBS) and, if not otherwise indicated, stained with TOTO-3 to a final concentration of 100 nM 5 to 15 minutes before measurement. KB cells were washed once with PBS and detached with 40 µg/mL collagenase 1A (crude collagenase from *Clostridium histolyticum*, 347 U/mg) in 40 mM sodium azide/0.73xPBS as recently described¹². After detachment, TOTO-3 was added to a final concentration of 100 nM. Analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm air-cooled argon ion laser and a 635-nm diode laser. The filter settings for emission were 530/30 nm bandpass (FL1) and 585/42 nm bandpass (FL2) for dyes excited by the 488-nm laser line, and a 661/20 nm bandpass (FL4) for dyes excited by the 635-nm laser line. Data acquisition was performed in linear mode and data were visualized in logarithmic mode. To discriminate between live and dead cells, cells incorporating high levels of TOTO-3 and showing a reduction in forward scatter as a measure for cell size were excluded from further analysis.

The ratio of FL1/FL4 values was calculated using the program FCSAssistant v1.3.1 (by Ray Hicks, Flow Cytometry Laboratory, Department of Medicine, University of Cambridge, Cambridge, UK). The calculated ratio was plotted in a histogram, setting the ratio of 1:1 at channel 200. The uptake ratio (r) was calculated by the following formula: $r = (\text{FL1/FL4 at } 37^\circ\text{C})/(\text{FL1/FL4 at } 4^\circ\text{C})$. A value of $r = 1$ represents no uptake.

Electron microscopy

Two micrograms of plasmid DNA in 50 μ L of 20 mM HEPES pH 7.4 were complexed with the indicated amount of (ligand)-PEI in 50 μ L HEPES and stained after 30 minutes incubation at RT with 1% uranyl acetate solution as described¹⁶. Microscopy was carried out on a Jeol 1200 Ex electron microscope (Jeol, Tokyo, Japan) at 80 kV.

Laser scanning microscopy

K562 cells were incubated with YOYO-1-labeled DNA/TfPEI₈₀₀ complexes. After incubation at 37°C, cells were washed with PBS and fixed with 4% paraformaldehyde (Fluka) in HBS at 4°C for at least 1 hour. Cells were pelleted at 150g and washed with 0.32 M sucrose in deionized water. To stain the cytoplasm membrane, cells were incubated with DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate, 5 μ M in 0.32 M sucrose) for 10 minutes at RT. After washing with 0.32 M sucrose, the pellet was reconstituted with water and 20 μ L of the cell suspension containing approximately $0.5-1 \times 10^5$ cells were put onto a slide, dried in the dark, mounted in Vectashield mounting medium (Vector laboratories, Burlingame, CA), and viewed on a Leica TCSNT laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon/krypton mixed gas laser delivering light at 488 nm, 568 nm, and 647 nm. YOYO-1 fluorescence (DNA/YOYO-1/PEI complexes) was excited with the 488-nm line; emission was collected using a 525/50 bandpass filter. The lipid-specific dye DiD was simultaneously excited with the 647-nm line, and emission was collected using a 660-nm long pass (zoom, 2; objective, 63 x oil; section size, 150 nm; 80-100 sections). Image processing and deconvolution was performed using a measured point spread function (220-nm beads, Polysciences, Warrington, PA) with the programs Huygens (SVI, Hilversum, the Netherlands) and Imaris (Bitplane AG, Zurich, Switzerland).

Cell transfections

For transfection studies, K562 or Jurkat cells were seeded in 48 or 96 well plates (Costar) 1 to 3 hours before incubation with complexes. Twenty-four hours after transfection, cells were harvested

and assayed for gene expression. For measuring luciferase gene expression, cells were lysed in 250 mM TRIS pH 7.3, 0.5% Triton X-100, and luciferase activity was measured for 10 seconds from an aliquot of the supernatant using a Clinilumat LB9507 instrument (Berthold, Bad Wildbad, Germany). For measuring Enhanced Green Fluorescent Protein (EGFP) expression, cells were washed in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson) using a modified protocol as recently described¹⁷. Transfections were carried out in duplicate.

RESULTS

Rapid and specific uptake of Tf-containing DNA/PEI₈₀₀ complexes

K562 cells were incubated with fluorescently labeled DNA/PEI₈₀₀ or DNA/Tf-PEI₈₀₀ complexes at either 37°C or 4°C. YOYO-1 (measured in the FL1 channel of the cytometer used), a DNA intercalating dye, was used to label the DNA. After harvesting, cells were stained with TOTO-3 (measured in the FL4 channel), another intercalating fluorophore with emission/excitation wavelengths different from those of YOYO-1. The cell-impermeable stain TOTO-3 binds only to DNA complexes accessible at the surface of the cell. The ratio of YOYO-1/TOTO-3 fluorescence (FL1/FL4) was calculated, representing the ratio of fluorescence of total associated DNA versus extracellularly bound DNA. The *r* value (uptake ratio of total versus cell surface-associated DNA) was obtained by dividing the median value of FL1/FL4 ratios obtained at 37°C by the value obtained at 4°C (no internalization). When incubating K562 cells with Tf containing DNA/PEI₈₀₀ complexes, a rapid internalization can be observed compared with that of the corresponding ligand-free complexes (Figure 1). A high degree of internalization of DNA/Tf-PEI₈₀₀ complexes is found after a total incubation time of 1 hour, whereas DNA/PEI₈₀₀ complexes are weakly internalized. Increasing the incubation time for up to 4 hours, Tf-containing complexes are further internalized, whereas DNA/PEI₈₀₀ complexes showed no enhanced uptake (data not shown). To test the specificity of Tf-mediated uptake, competitive inhibition was performed with free Tf. The presence of free Tf reduces the uptake to a level obtained with DNA/PEI₈₀₀

complexes. As shown in Table 1, this correlates well with gene expression. The enhanced gene expression achieved with Tf-containing PEI₈₀₀ can be reduced to a level achieved with Tf-free complexes by adding free Tf.

Influence of particle size on receptor-mediated endocytosis

Receptor-mediated endocytosis of transferrin is accompanied by the formation of clathrin-coated vesicles with an average diameter of approximately 100 nm¹⁹; thus, a size limitation should exist for particles that are taken up by receptor-mediated endocytosis. To our surprise, we recently found that aggregated DNA/Tf-PEI₈₀₀ complexes generated in HBS with an average size greater than 500 nm resulted in more efficient gene transfer than did small particles formed in HBG¹⁷ (see also Table 1). We then compared these 2 types of complexes using the flow cytometric assay (Figure 2). Both the total (FL1) and the cell surface association (FL4) of the large particles was higher than those of the small particles (Figure 2A). Using the large particles, however, no rapid internalization of Tf-containing complexes (as would be presented by a high *r* value) was found (Figure 2B) (even when increasing the incubation time up to 4 hours; data not shown). As confirmed by laser scanning microscopy, large, aggregated complexes mostly stick on the cell surface and are only sparsely internalized; however, with the small DNA/Tf-PEI₈₀₀ complexes, an almost complete internalization can be observed (Figure 2C). In contrast, gene expression follows an opposite pattern (Table 1). Although the relative internalization is far less efficient with large aggregates, the resulting gene expression is 4 (PEI₈₀₀) to 11 (Tf-PEI₈₀₀) times higher than that of small aggregates.

Usage of PEI25 conjugates

Abdallah et al²⁰ have shown that gene expression after direct application of polyplexes into the brain is even higher using PEI₂₅ instead of PEI₈₀₀. Our own studies on applying DNA/PEI complexes systemically into mice showed that under certain conditions PEI₂₅ caused less acute

Figure 1. Tf-mediated internalization of DNA/transferrin (Tf)- polyethylenimine (PEI)₈₀₀ complexes into K562 cells. K562 cells (2×10^5 cells in 0.2 mL medium) were incubated for 30 minutes at either 37°C or 4°C with (A) YOYO-1 labeled DNA/PEI₈₀₀ or (B) DNA/Tf-PEI₈₀₀ complexes (N/P 4.8) generated in HBG (1.5 µg DNA in 75 µL) and, after replacing the medium, for another 30 minutes. Harvesting, staining with TOTO-3, and analyzing cells were carried out as described in the Materials and Methods section of this article. Competitive inhibition was performed in the presence of 5 mg/mL Tf during the first 30 minutes of incubation (C: PEI₈₀₀, D: Tf-PEI₈₀₀). Density plots of cells incubated at 37°C are shown. The *r* value (uptake ratio) shown was obtained by dividing the median value of FL1/FL4 ratios obtained at 37°C by the value obtained at 4°C (no internalization).

Table 1. Specificity of Transfection

Transfection Conditions	RLU x 10 ³ /500 000 cells
HBG (80-150 nm):	
DNA/PEI ₈₀₀	1.82 +/- 0.13
DNA/PEI ₈₀₀ + Tf	0.79 +/- 0.51
DNA/Tf-PEI ₈₀₀	47.23 +/- 1.98
DNA/Tf-PEI ₈₀₀ + Tf	1.91 +/- 0.25
HBS (> 500 nm):	
DNA/PEI ₈₀₀	6.59 +/- 1.25
DNA/PEI ₈₀₀ + Tf	1.61 +/- 0.00
DNA/Tf-PEI ₈₀₀	526.21 +/- 72.7
DNA/Tf-PEI ₈₀₀ + Tf	2.54 +/- 2.08

HBG indicates HEPES buffered glucose (20 mM HEPES pH 7.4, 5% glucose wt/vol); HBS, HEPES buffered saline (20 mM HEPES pH 7.4, 150 mM NaCl); PEI, polyethylenimine; RLU, relative light units Tf, transferrin. 5×10^5 K562 cells in 0.5 mL medium were transfected for 4 hours with the indicated complex (containing 5 µg DNA) mixed in either HBG or HBS (N/P 6) in the presence or absence of 5 mg/mL Tf. Twenty-four hours after transfection, cells were harvested and assayed for luciferase expression as described. The data in brackets indicate the size range of the particles used. The complex size was measured as described in Blessing et al¹⁶.

Figure 2. Influence of complex size on internalization of K562 cells. K562 cells were incubated as described in [Figure 1](#); complexes (DNA/Tf-PEI₈₀₀) were generated at an N/P ratio of 4.8 either in HBS (large complexes) or HBG (small complexes). (A) Histogram plots for FL1 (total associated DNA, left graph) and FL4 (surface-bound DNA, right graph) of cells incubated with the indicated complexes at 37°C. Dotted line indicates mock-treated cells; bold line, cells incubated with complexes generated in HBS; narrow line, cells incubated with complexes generated in HBG. (B) FL1/FL4 uptake ratios of cells incubated at either 37°C (bold line) or 4°C (narrow line) with complexes generated in HBG (left) or HBS (right), the r value is shown for the individual graphs. (C) Laser scanning microscopy of K562 cells incubated with YOYO-1 labeled DNA/Tf-PEI₈₀₀ complexes. K562 cells were incubated with DNA/Tf-PEI₈₀₀ complexes in either HBG (left) or HBS (right) for 30 minutes at 37°C. After washing, cells were fixed and viewed with a laser scanning microscope as described in the Materials and Methods section. Central sections of the cells are shown. Green indicates YOYO-1 labeled DNA complexes; red, DiD fluorescence.

Figure 4. Cell binding, internalization, and gene transfer with Tf-PEI₂₅. 2×10^5 K562 cells in 0.2 mL medium were incubated with DNA/PEI₂₅ /Tf-PEI₂₅ complexes generated in HBG (1.5 µg YOYO-1 labeled DNA complexed with total 1.125 µg (Tf)-PEI₂₅, N/P 6.0) for 30 minutes and, after replacing the medium, for another 30 minutes. PEI₂₅, Tf-PEI₂₅, or a mixture of PEI₂₅ and Tf-PEI₂₅ (113 ng Tf-PEI₂₅ + 1.013 µg PEI₂₅ for 1/10, 56 ng Tf-PEI₂₅ + 1.069 µg PEI₂₅ for 1/20) were used for DNA complexation. Cells were incubated at either 37°C or 4°C. Staining with TOTO-3 and analysis were carried out as described in the Materials and Methods section. (1A) Total cellular association at 37°C or uptake ratios (r) are shown; $n = 4 \pm$ SD. (B) For transfection studies, 5×10^4 K562 cells in 50 µL medium were transfected with the same complex formulation (containing 0.5 µg DNA and in total 375 ng [Tf]-PEI₂₅). Media was replaced after 30 minutes and luciferase gene expression measured after 24 hours as described; $n = 2 \pm$ SD. Data from a representative experiment are shown.

Figure 3. (left) Electron microscopy of DNA/(ligand)-PEI₂₅ complexes. Two micrograms of DNA in 50 µL were complexed with the following amounts of (ligand)-PEI₂₅ (N/P 6) in 50 µL as described in the Materials and Methods section: (A) 1.5 µg PEI₂₅; (B) 1.5 µg Tf-PEI₂₅; (C) 1 µg PEI₂₅ + 0.5 µg Tf-PEI₂₅; and (D) 1.5 µg EGF-PEI₂₅. The bar represents 200 nm in A, C, and D; 500 nm in B.

toxicity compared with PEI₈₀₀¹⁵. To use PEI₂₅ for receptor-mediated gene transfer, the properties of Tf-, EGF-, and antiCD3-PEI₂₅ conjugates were studied in terms of DNA condensation, cellular uptake, and reporter gene expression. Electron microscopy revealed that Tf-PEI₂₅ condensed DNA into large, fibrous aggregates of up to micrometer size (Figure 3B) and condensation into spherical complexes, as observed for DNA/PEI₂₅ (Figure 3A), is hindered. Because of these properties, we used different mixtures of ligand-PEI₂₅ and PEI₂₅ to condense the DNA. Replacing at least two-thirds of the Tf-PEI₂₅ with unmodified PEI₂₅ enabled DNA condensation as shown with PEI alone (Figure 3C). A similar effect could be found using a monoclonal antiCD3 antibody coupled to PEI: The antiCD3-PEI₂₅ conjugate formed large aggregates, whereas optimized mixtures with unmodified PEI₂₅ condensed the DNA into spherical particles (data not shown) and enabled their specific uptake into target cells (see following). Compared to the relatively large ligands Tf (80 kd) and antiCD3 (150 kd), the 8 kd protein EGF did not interfere with PEI-mediated complex formation: The EGF-PEI₂₅ conjugate properly condensed DNA (Figure 3D) and enabled specific internalization (see following). Measuring total cellular association of complexes (YOYO-1 fluorescence in FL1), DNA complexed with Tf-PEI₂₅ is less associated with cells than is DNA/PEI₂₅, whereas a mixture of PEI₂₅ with Tf-PEI₂₅ results in the highest cellular association (Figure 4A). In terms of internalization, as expressed by the *r* value, a low degree of internalization is observed for DNA/PEI₂₅ complexes, whereas Tf-PEI₂₅ complexes and complexes containing only 5% to 10% Tf-PEI₂₅ (wt/wt, based on PEI) are efficiently internalized (Figure 4B). Nevertheless, although reporter gene expression using DNA/Tf-PEI₂₅ complexes is more than 20-fold higher than that of DNA/PEI₂₅, optimized Tf-PEI₂₅/PEI₂₅ mixtures could further enhance gene expression (Figure 4C). Antibody-mediated binding and internalization into Jurkat cells showed a similar pattern (Figure 5): DNA/PEI₂₅ complexes without antibodies bind to the cell surface but are not internalized (Figure 5B). Although total cellular associations using DNA condensed with antiCD3-

PEI₂₅ were higher compared with those of PEI₂₅ (Figure 5C), efficient internalization could be achieved only by partially replacing antiCD3-PEI₂₅ by unconjugated PEI₂₅ (Figures 5D and 5E). Competitive inhibition with unconjugated antiCD3 (140-fold molar excess) has shown the specificity of the uptake (data not shown). Using EGFP as a reporter gene, $12.5 \pm 0.9\%$ of EGFP-positive cells were observed with DNA/PEI₂₅/antiCD3-PEI₂₅ (at 20/1 wt/wt ratio of PEI₂₅/antiCD3-PEI₂₅), whereas ligand-free complexes mediated very low levels of gene expression ($0.1 \pm 0.01\%$).

Studying EGF-mediated internalization using the human adenocarcinoma cell line KB, an optimal uptake is already achieved with DNA/EGF-PEI₂₅ complexes (Figure 6), measuring both total cellular association (Figure 6A) and internalization rate (Figure 6B). Competitive inhibition with free EGF (100-fold molar excess) was performed that confirmed the specificity of EGF-mediated uptake.

Figure 5. Antibody-mediated binding and internalization on Jurkat cells. 2 ± 10^5 Jurkat cells in 0.2 mL medium were incubated for 30 minutes with complexes generated in HBG (N/P 6.0; 1.5 μ g YOYO-1 labeled DNA in 75 μ L) and, after replacing the medium, for another 30 minutes. Incubations were carried out with DNA complexed to (B) PEI₂₅, (C) antiCD3-PEI₂₅, (D) PEI₂₅/antiCD3-PEI₂₅ 3/1 wt/wt, or (E) PEI₂₅/antiCD3-PEI₂₅ 20/1 wt/wt. Control cells (no complexes) are shown in 5A. Staining with TOTO-3 and analysis was carried out as described in the Materials and Methods section. Uptake ratios (*r*) for each condition are shown in the individual density plots.

Figure 6. EGF-mediated internalization into KB cells. 2×10^5 KB cells in 0.5 mL medium were incubated for 30 minutes with DNA/PEI₂₅ or DNA/EGF-PEI₂₅ complexes (N/P 6.0) generated in HBG (3 μ g YOYO-1 labeled DNA in 150 μ L) and, after replacing the medium, for another 30 minutes. Competitive inhibition was carried out with 100-fold molar excess of EGF during the first half-hour of incubation. (A) Total cellular association (FL1, YOYO-1 fluorescence). (B) Uptake ratio (r); $n = 2-5 \pm$ SD.

Influence of covalently bound PEG on receptor-mediated internalization

Recently we described the covalent coupling of PEG to DNA/PEI₈₀₀ complexes with a succinimidyl derivate of PEG through the primary amino groups. By selecting the appropriate ratio of PEG to PEI (PEG/PEI 10/1 wt/wt), the in vivo properties for systemic gene delivery of DNA/Tf-PEI₈₀₀/PEG complexes are significantly improved, whereas the total gene-transfer efficiency in vitro is not negatively affected¹⁸. As a result, we evaluated the effect of covalently bound PEG to DNA/ligand-PEI complexes on ligand-mediated internalization. Using moderate amounts of PEG (PEG/PEI ratio 5/1-15/1), Tf-enhanced uptake of particles can still be achieved (Figure 7A). A further increase in the amount of PEG used (30/1 ratio) reduced the uptake ratio to a level that is obtained with DNA/PEI complexes. In terms of total cellular association, low amounts of PEG used only moderately decreased FL1 (data not shown), whereas the highest ratio of PEG/PEI caused a

Figure 7. Influence of PEG on Tf-mediated binding and internalization on K562 cells. 2×10^5 K562 cells were incubated in 0.2 mL medium with 75 μ L complex generated in HBG (N/P 6.0, 1.5 μ g YOYO-1 labeled DNA) as described in Figure 1. (A) Complexes used have been PEGylated with the indicated wt/wt ratios of PEG/PEI as described in the Materials and Methods section. The uptake ratio (37°C versus 4°C) was calculated as described and plotted on the graph. Full bars indicate DNA/Tf-PEI₈₀₀; blank bars, DNA/PEI₈₀₀. $n = 2 \pm$ SD. (B) K562 cells were incubated with DNA/Tf-PEI₈₀₀ complexes as described in (A). Total cellular association measured in FL1 is shown. Dotted line indicates control cells; bold line, cells incubated with DNA/Tf-PEI₈₀₀ or DNA/Tf-PEI₈₀₀/PEG; narrow line, PEG/PEI 30/1 wt/wt.

significant decrease (Figure 7B). These data are also consistent with previous observations concerning gene expression in K562 cells¹⁸: PEG/PEI ratios up to 15/1 do not interfere with Tf-enhanced gene transfer, whereas higher ratios do (ie, a 100-fold reduction in luciferase expression with a PEG/PEI ratio of > 20/1; M.O., unpublished observations). In the case of KB cells, even the highest PEG/PEI ratio of 30/1 only slightly decreased internalization of DNA/PEI₂₅ and DNA/EGF-PEI₂₅, whereas internalization of DNA/Tf-PEI₂₅ complexes (optimized PEI₂₅/Tf-PEI₂₅ ratio 20/1 wt/wt) was significantly affected (Figure 8A). PEGylation of untargeted (Figure 8B) and Tf-containing complexes (Figure 8C) decreased total cellular association; DNA/EGF-PEI₂₅ complexes remained unaffected (Figure 8D).

Figure 8. Influence of PEG on complex binding and internalization on KB cells. 2×10^5 KB cells in 0.5 mL medium were incubated with 150 μ L (N/P 6.0, 3 μ g YOYO-1 labeled DNA) complex: DNA/PEI₂₅, DNA/PEI₂₅/Tf-PEI₂₅ = 20/1 wt/wt or DNA/EGF-PEI₂₅. After 30 minutes, the medium was replaced, and cells were incubated for another 30 minutes. Afterward, cells were harvested, stained with TOTO-3, and analyzed as described in the Materials and Methods section. (A) Uptake ratios for the indicated complexes, either non-PEGylated (full bars) or PEGylated with a PEG/PEI wt/wt ratio of 30/1 (clear bars) are shown; $n = 2 \pm$ SD. (B-D) Total cellular association measured in FL1 (YOYO-1) is shown. Dotted line indicates control cells; bold line, cells incubated with non-PEGylated complexes; narrow line, PEGylated complexes PEG/PEI 30/1 wt/wt. (B) DNA/PEI₂₅. (C) DNA/PEI₂₅/Tf-PEI₂₅. (D) DNA/EGF-PEI₂₅.

DISCUSSION

Gene delivery via receptor-mediated endocytosis has been shown to be a powerful method for the specific delivery of genes to certain cell types or tissues. We and several other groups have described ligand-mediated gene transfer [2,5,7, 9,14, 21-24](#). Studies of receptor-dependent uptake of the ligand-containing complexes included binding studies of the DNA complex to the target receptor; applying fluorescence microscopy to monitor binding and uptake at 4°C (no endocytosis) and 37°C (endocytosis) using labeled DNA complexes; testing whether gene expression is reduced in competition experiments with free ligand or with DNA complexes lacking the ligand and whether it is low in cells without receptor; and testing whether transfection

efficiency is enhanced after up-regulation of the cellular receptor [25](#). Because the size of endocytic vesicles is approximately 100 nm²⁶, gene transfer particles should be small enough to be taken up by this mechanism. The time range for the uptake of ligands is several minutes, as shown for Tf²⁷ and EGF [28](#), or up to 30 minutes in the case of antiCD3 [29](#). The current work describes several parameters that influence receptor-mediated uptake and gene expression of DNA/PEI complexes. To study the internalization parameters of polyplexes, we applied a novel flow-cytometry assay to characterize ligand-mediated endocytosis of DNA/ligand-PEI complexes¹². We found that K562 cells rapidly take up small DNA/PEI₈₀₀ complexes containing Tf (covalently coupled to PEI₈₀₀) as an internalizing ligand. Uptake is specific for Tf, as competition experiments with free Tf have shown. In contrast, when using large, aggregated complexes, the large particles mainly stick on the cell surface. Nevertheless, with regard to gene transfer efficiency, aggregated DNA/Tf-PEI₈₀₀ shows a higher luciferase gene-transfer level compared to that of the smaller ones generated under low ionic conditions, as described previously¹⁷. The higher transfection activity can be explained by 2 mechanisms: 1) the total cell association of aggregates is significantly higher than that of the small ones, and 2) the endosomolytic activity of aggregates is far higher than that of 40- to 100-nm complexes¹⁷.

Although Tf-PEI₈₀₀ has been shown to be a powerful transfection agent both in vitro^{7, 17} and in vivo^{18, 30}, the high molecular weight PEI can cause acute toxicity in mice when applied systemically. In contrast, using the lower molecular weight PEI₂₅ at a low N/P ratio (N/P 5 or below) or linear PEI 22 kd, no toxicity was observed [15, 31](#). Therefore, we also evaluated the performance of ligand-PEI₂₅ conjugates in terms of DNA condensation, cellular uptake, and transfection efficiency. Using large proteins (Tf, 80 kd; OKT3, IgG, approximately 150 kd) conjugated to the relatively small PEI molecule (25 kd), the bulky protein molecule hinders proper condensation into small DNA/ligand/PEI₂₅ particles and also results in suboptimal gene transfer efficiency. In contrast, smaller molecules

like EGF (see following) or peptides¹⁴ do not interfere with DNA condensation. Erbacher et al³² obtained similar results when modifying PEI₂₅ with PEG containing terminal galactose residues: Wormlike, extended structures were observed, and gene transfer was reduced compared to nonmodified PEI₂₅. Conjugates of Tf with PEI₈₀₀ enabled condensation of DNA into small particles at least up to Tf/PEI ratios of 8/1 M/M¹⁷, which corresponds to a 1/1 wt/wt ratio in the case of the 8/1 conjugate. The rationale, therefore, was to partially replace Tf-PEI₂₅ with unmodified PEI₂₅ to obtain similar wt/wt ratios. Indeed, when replacing at least 67% of Tf-PEI₂₅ by PEI₂₅ (final Tf/PEI₂₅ wt/wt ratio in the complex of 1/1), DNA was properly condensed and maximal gene transfer efficiency was obtained with 5% to 15% Tf-PEI₂₅ combined with 90% to 95% PEI₂₅. Most recently, Kircheis et al¹⁵ used this approach for targeted gene delivery to tumors after systemic application where the attached Tf shields the positive complex surface charge and allows circulation in the bloodstream. Although internalization higher than that of PEI₂₅ alone was already achieved with Tf-PEI₂₅ alone, no internalization was achieved using DNA/antiCD3-PEI₂₅ complexes on Jurkat cells, and only the partial replacement of antibody-PEI₂₅ by PEI₂₅ enabled internalization and gene expression.

Both K562 and Jurkat cells are lymphocyte-derived cell lines that grow in suspension and only sparsely internalize untargeted DNA complexes^{12,33}. In contrast, adherent cell lines have been reported to rapidly internalize gene transfer complexes bearing a positive surface charge³³, mostly through interaction with membrane-associated proteoglycans³⁴. Our results show that DNA/PEI complexes bind efficiently to KB cells and that after 1 hour a high uptake ratio (total versus cell surface associated DNA, *r* value of 2) is obtained. Nevertheless, when using EGF as a targeting ligand, both binding and internalization was even further enhanced. These observations are in line with recent experiments in our laboratory, where an up to 300-fold increased luciferase expression could be achieved with EGF-containing complexes¹⁶.

We used PEG (molecular weight, 5000 d) covalently coupled to the surface of DNA/Tf-

PEI₈₀₀ complexes to reduce interaction with blood components after systemic application in vivo. The half-life of the complexes in blood was increased and gene transfer into distant tumors was achieved¹⁸. Using a PEG/PEI wt/wt ratio of 10/1 (molar ratio succinimidylester/primary amino groups 1/3), gene transfer in vitro was not negatively affected. When further increasing the PEG/PEI ratio, a reduction in the gene transfer efficiency with DNA/Tf-PEI complexes was observed. With a PEG/PEI ratio of 30/1 wt/wt (molar ratio succinimidylester/primary amino groups 1/1), Tf-mediated gene transfer is reduced to a level obtained with DNA/PEI complexes. Studying cellular binding and internalization of DNA/Tf-PEI complexes, a 15/1 wt/wt ratio of PEG/PEI only slightly reduced internalization, whereas a further increase in the rate of PEG modification reduced both cell binding and internalization to a level achieved with untargeted complexes. Apparently, there is a correlation between cell binding, internalization, and reporter gene expression. Two effects might cause decreased uptake by excessive PEGylation: 1 possibility is the hiding of the protein molecule within a layer of PEG on the surface on the cell. A layer of PEG (molecular weight, 5 kd) on liposomes has been described to have a thickness of 6 nm³⁵. According to the literature³⁶, Tf has dimensions of approximately 10 x 5 nm. Therefore, a more probable explanation is the modification of the primary amino groups within the proteins by the PEG derivative (M-PEG-SPA). The results obtained by comparing Tf- and EGF-mediated internalization on KB cells support the second possibility: Without PEGylation, a significantly faster internalization is achieved with both Tf- or EGF-containing complexes compared with that of the ligand-free DNA/PEI particles. A high degree of PEGylation (30/1) significantly reduces internalization of DNA/Tf-PEI complexes, whereas EGF-mediated uptake is less affected. Although the differences were less pronounced than observed with K562 cells, total cellular association after PEGylation was slightly reduced with untargeted and Tf-targeted complexes, whereas binding of DNA/EGF-PEI₂₅ complexes was not affected. Despite its small size (8 kd), the ability of complex-bound EGF to interact with the cellular EGF-receptor is not

hindered by the PEG layer on the complex surface. Also gene expression is only slightly reduced when PEGylating DNA/EGF-PEI complexes, but the reporter gene expression (luciferase) is still 2 orders of magnitude higher than that of simple DNA/PEI complexes¹⁶. In contrast to Tf³⁷, EGF³⁸ contains no lysine in the primary amino acid sequence. Using the succinimidyl derivative of PEG, binding can only occur to the primary amino groups of PEI and not to the EGF molecule (the only primary amino group in the protein is already modified by coupling to PEI).

CONCLUSION

In summary, many factors can influence ligand-mediated gene transfer of DNA/polycation complexes. In this work, strategies to formulate targeted gene delivery particles (ie, with an optimal amount of ligands and degree of surface modification with PEG) are presented. As demonstrated by several methods, the complex size, molecular weight of polycation, ligand density, and PEGylation have important relevance for the design of targeted gene transfer vectors.

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